Degradation of HER2/*neu* by apigenin induces apoptosis through cytochrome *c* release and caspase-3 activation in HER2/*neu*-overexpressing breast cancer cells

Tzong-Der Way[a], Ming-Ching Kao[b], Jen-Kun Lin[a,*]

[a] *Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-ai Road, Taipei 10018, Taiwan*

[b] *Department of Biochemistry, School of Medicine, China Medical University, Taichung 404, Taiwan*

Received 23 September 2004; revised 12 November 2004; accepted 15 November 2004

Available online 30 November 2004

Edited by Veli-Pekka Lehto

Abstract **We have shown that exposure of the HER2/*neu*-overexpressing breast cancer cells to apigenin resulted in induction of apoptosis by depleting HER2/*neu* protein and, in turn, suppressing the signaling of the HER2/HER3-PI3K/Akt pathway. Here, we examined whether inhibition of this pathway played a role in the anti-tumor effect. The results revealed that treatment with apigenin induced apoptosis through cytochrome *c* release and caused a rapid induction of caspase-3 activity and stimulated proteolytic cleavage of DFF-45. Furthermore, apigenin downregulated cyclin D1, D3 and Cdk4 and increased p27 protein levels. Colony formation in the soft agar assay, a hallmark of the transformation phenotype, was preferentially suppressed in HER2/*neu*-overexpressing breast cancer cells in the presence of apigenin. In addition, a structure–activity relationship study indicated that (1) the position of B ring; and (2) the existence of the 3′, 4′-hydroxyl group on the 2-phenyl group were important for the depletion of HER2/*neu* protein by flavonoids. These results provided new insights into the structure–activity relationship of flavonoids.**

Keywords: Apigenin; HER2/*neu*; Caspase-3; Cyclin D1; Flavonoids

1. Introduction

Flavonoids may be considered to be potential compounds for selectively blocking signal transduction pathways. Based on their skeleton, flavonoids are classified into eight groups: flavans, flavanones, isoflavanones, flavones, isoflavones, anthocyanidines, chalcones and flavonolignans. Epidemiological studies have shown that the consumption of vegetable, fruits and tea is associated with a decreased risk of cancer and cardiovascular diseases, and flavonoids are believed to play an important role in preventing these diseases [1]. Flavonoids in human diet may reduce the risk of various cancers [2], especially hormone-dependent breast and prostate cancers, and prevent menopausal symptoms. These data indicate that certain flavonoids may be used as possible chemoprotective or chemotherapeutic agents.

Breast cancer is the most frequently diagnosed malignancy among women, with an approximate incidence of one million new cases worldwide annually. Two genes with particular significance to this disease are *erbB2* (*HER2*/*neu*) and *cyclin D1*. Both genes have prognostic significance because they are frequently overexpressed and implicated in experimental models of breast cancer [3,4]. The HER2/*neu* oncogene, the second member of the epidermal growth factor (EGF) receptor family, encodes a transmembrane receptor protein tyrosine kinase. Overexpression of HER2/*neu*, which has been seen in approximately 30% of breast cancers, is associated with poor overall survival [5]. In particular, it has been found to be associated with increased metastatic potential and resistance to chemotherapeutic agents. Interactions between HER2/*neu* and cyclin D1 appear to have therapeutic relevance, because the anti-HER2/*neu* monoclonal antibody trastuzumab (Herceptin) reduces cyclin D1 protein levels [6]. In addition, flavopiridol inhibits CDK activity, lowers cyclin D1 levels [7], and reduces HER2/*neu* receptor levels [8]. Cyclin D1 functions were first shown to be downstream of HER2/*neu*-mediated transformation in tissue culture models. In murine models, cyclin D1-deficient mice were completely resistant to erbB2-mediated mammary tumorigenesis [9]. The combination of these findings in both mouse models and in human specimens would be a stringent test for the idea that cyclin D1 is downstream of, and required for, the HER2/*neu*-mediated tumorigenesis.

Apoptosis, a genetically regulated form of cell death, is one of the important pathways through which chemotherapeutic agents inhibit the growth of cancer cells [10]. It can be triggered by various extracellular and intracellular stimuli that result in coordinated activation of a family of cysteine proteases called caspases. In response to certain apoptotic stimuli, cytochrome *c* is released from mitochondria [11,12] into the cytoplasm, where it has an entirely different function. The released cytochrome *c* induces the formation of a caspase activation complex with Apaf-1, which directly binds the zymogen form of caspase-9 via a homophilic interaction involving CARD (the caspase recruitment domain) [13]. The CARD is a region of highly conserved residues in the amino terminus of molecules that are recruited for complex signaling during apoptosis. Activated caspase-9 can proteolytically process the zymogen form of downstream caspases, such as caspase-3 [14].

*Corresponding author. Fax: +886 2 2391 8944.
E-mail address: jklin@ha.mc.ntu.edu.tw (J.-K. Lin).

0014-5793/$22.00
doi:10.1016/j.febslet.2004.11.061

Among the plant flavonoids, apigenin (4′,5,7,-trihydroxyflavone) is a chemopreventive compound and an inhibitor of certain signal transduction pathways [15]. We have shown that apigenin induces apoptosis by depleting HER2/*neu* protein in HER2/*neu*-overexpressing MDA-MB-453 breast cancer cells via proteasomal degradation. Furthermore, the inhibition of cell growth and induction of apoptosis by apigenin may be through suppression of HER2/HER3 signaling and disruption of the PI3K/Akt-dependent pathway [16]. In this study, we examined whether the signaling-pathways downstream of HER2/*neu* that regulate cell cycle progression and/or cell death are modified by apigenin. Moreover, we investigated the inhibitory action of 19 flavonoids of four chemical classes on the degradation of HER2/*neu* protein, to identify the structural requirements of flavonoids necessary for modulating the potency of the degradation of HER2/*neu* protein.

2. Materials and methods

2.1. Chemicals

Flavone, 5-methoxyflavone, 7,8-dihydroxyflavone, apigenin, kaempferol, quercetin, myricetin, rutin, and genistein were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chrysin, luteolin, galangin, fisetin, pinocembrin, naringenin, isosakuranetin, eriodictyol, hesperetin, and biochanin A were purchased from Extrasynthese Inc. (Genay, France).

2.2. Cell culture

The human breast cancer cell lines used in this study were MDA-MB-453, BT-474, and SKBr-3, all of which overexpress HER2/*neu*, and MCF-7 and T47-D which express the basal level of HER2/*neu*. We also used HBL-100 cell line, which is derived from a normal human breast tissue transformed by SV40 large T antigen and expresses a basal level of HER2/*neu*. All of the cells were grown in DMEM/F12 (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) and gentamicin (50 mg/ml). Cells were grown in a humidified incubator at 37 °C under 5% CO_2.

2.3. Caspase activity

After treatment, cells were collected and washed with PBS and resuspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol (DTT), 2 mM PMSF, 10 mg/ml pepstatin A and 10 mg/ml leupeptin. Cell lysates were clarified by centrifugation at 12 000 rpm for 15 min and the clear lysates containing 150–500 μg of protein were incubated with 2.5 mM substrate Ac-DEVD-AMC at 30 °C for 1 h. Levels of released 7-amino-4-methyl coumarin (AMC) were measured using a spectrofluorometer (Hitachi F-4500) with excitation at 360 nm and emission at 460nm (Promega's CaspACE2 Assay System, Madison, WI, USA).

2.4. Cytochrome c release

Mitochondrial and cytosolic fractions were prepared by resuspending cells in ice-cold buffer A [250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 mg/ml PMSF, 8 mg/ml aprotinin and 2 mg/ml leupeptin (pH 7.4)). Cells were passed through a 27G-needle 10 times. Unlysed cells and nuclei were pelleted by centrifugation for 10 min at $750 \times g$. The supernatant was then centrifuged at $100\,000 \times g$ for 15 min. The pellet was resuspended in buffer A and represents the mitochondrial fraction. The supernatant was centrifuged again at $100\,000 \times g$ for 1 h. The final supernatant represents the cytosolic fraction.

2.5. Western blot analysis

Cells (1.5×10^6) were seeded onto a 100-mm tissue culture dish containing 10% FBS DMEM/F12 and cultured for 24 h. Then, cells were incubated in 1% FBS DMEM/F12 treating with various dosees of apigenin for various time periods. Cells were washed three times with PBS and then lysed in gold lysis buffer (10% glycerol, 1% Triton X-100, 137 mM NaCl, 10 mM NaF, 1 mM EGTA, 5 mM EDTA, 1 mM sodium pyrophosphate, 20 mM Tris–HCl, pH 7.9, 100 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.1% sodium dodecyl sulfate, 10 μg/ml aprotinin, 1 mM PMSF, and 10 μg/ml leupeptin). Protein content was determined against a standardized control, using the Bio-Rad protein assay kit (Bio-Rad Laboratories). A total of 50 μg of protein was separated by SDS–PAGE and transferred onto nitrocellulose filter (NCF) paper (Schleicher & Schuell, Inc., Keene, NH). Non-specific binding to the NCF paper was minimized with a blocking buffer containing non-fat dried milk (5%) and Tween 20 (0.1%, v/v) in PBS (PBS/Tween 20). Then, the NCF paper was incubated with primary antibodies followed by the horseradish peroxidase-conjugated goat anti-mouse antibody (1:2500 dilution; Boehringer Mannheim Corp., Indianapolis, IN). Reactive bands were visualized with an enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL). The intensity of the bands was scanned and quantified with NIH image software.

2.6. Soft agar colony formation assay

The effects of apigenin on the soft agar colony formation of various human breast cells were investigated. Briefly, cells (1×10^4) were seeded in 6-cm culture dish containing 0.35% low-melting agarose over a 0.7% agarose layer in the presence of varying concentrations of apigenin or control vehicle and incubated for 3 weeks at 37 °C. Colonies were then stained with *p*-iodonitrotetrazolium violet (1 mg/ml) and those colonies larger than 100 μm were counted.

3. Results

3.1. Apigenin-induced cytochrome c release and caspase-3 activation

The release of cytochrome *c* from the mitochondria is the central gate in turning on apoptosis. This release is regulated by the interaction of proapoptotic proteins, including Bid, Bax and Bak, and antiapoptotic proteins, including Bcl-2 and Bcl-XL, as well as a specific class of inhibitors of apoptosis proteins, including Akt, survivin, and heat-shock proteins [17]. In our previous report [16], we found that apigenin inhibits Akt kinase activity in HER2/*neu* -overexpressing breast cancer cells. We next tested whether apigenin also induced cytochrome *c* release. Fig. 1A clearly shows that apigenin led to the release of cytochrome *c* into the cytosol in a dose-dependent manner. Subsequent to the release of cytochrome *c*, caspases play a central role in mediating various apoptotic responses. To monitor the enzymatic activity of caspases during apigenin-induced apoptosis, we used specific fluorogenic peptide substrate (Ac-DEVD-AMC) for caspase-3, and found that apigenin induced caspase-3 activity in a dose- and time-dependent manner (Fig. 1B). An inhibitor of caspase-3 protease, Z-DEVD-FMK, inhibited apigenin-stimulated caspase-3 activity (Fig. 1C). Western blotting results also suggest that apigenin increases caspase-3 levels by increasing the cleavage of the caspase-3 precursor pro-caspase-3 in a time- and dose-dependent manner (Fig. 1D). The above results clearly indicate that caspase-3 protease is activated in response to apoptosis induced by apigenin.

3.2. Apigenin treatment causes the cleavage of DFF-45, an inhibitor of endonuclease

DFF, a DNA fragmentation factor, has been identified as a heterodimeric protein that triggers DNA fragmentation during apoptosis although DFF has no nuclease activity [18]. Caspases activated by apoptotic signals cleave DFF-45 to release caspase-activated deoxyribonuclease [19,20]. Once caspase-

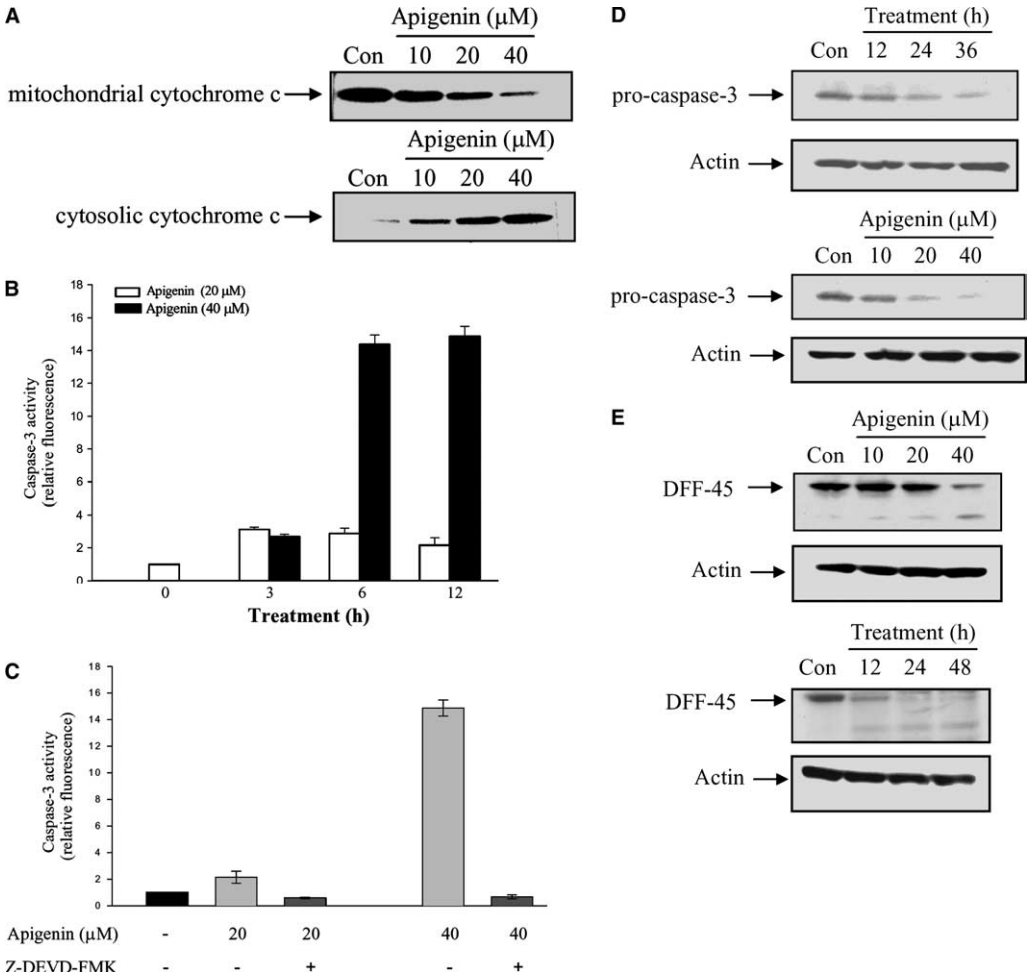


Fig. 1. Apigenin-induced cytochrome *c* release, caspase-3 activation and DFF-45 cleavage. (A) MDA-MB-453 cells were incubated with DMSO (Con) or apigenin (10, 20 and 40 μM) at 37 °C for 24 h. Levels of cytochrome *c* in the mitochondrial (upper panel) and cytosolic (lower panel) fraction were analyzed by immunoblotting. (B) MDA-MB-453 cells were incubated with apigenin (20 and 40 μM) at 37 °C for various times and cell lysates were analyzed for caspase-3 activity. Columns represent means for three independent experiments and are expressed relatively to 0 h. Bars represent S.E. (C) MDA-MB-453 cells were pretreated for 1 h with a caspase inhibitor (Z-DEVD-FMK) prior to the addition of 20 or 40 μM apigenin for 12 h, and cell lysates were analyzed for caspase-3 activity. Columns represent means of three independent experiments and are expressed relatively to 0 h. Bars represent S.E. (D) MDA-MB-453 cells were incubated with DMSO (Con) or apigenin (20 μM) at 37 °C for various times (upper column) and for 24 h at various doses (lower column). Levels of pro-caspase-3 and actin were analyzed by immunoblotting. (E) MDA-MB-453 cells incubated with DMSO (Con) or apigenin (40 μM) at 37 °C for various times (lower column) and various doses for 48 h (upper column). Levels of DFF-45 and actin were analyzed by immunoblotting.

activated deoxyribonuclease is released, caspase-3 cleaves DFF-45. Then, the cleaved DFF-45 can enter the nucleus where it degrades chromosomal DNA [18]. We further explored the possibility that activation of caspase-3 may also induce DFF-45 protein degradation. Indeed, treatment of MDA-MB-453 cell lines with apigenin caused proteolytic cleavage of DFF-45 in a dose- and time-dependent manner (Fig. 1E).

3.3. Apigenin alters cell-cycle regulatory proteins in HER2/*neu*-overexpressing breast cancer cells

When Akt is active, it phosphorylates an increasing number of substrates involved in apoptosis, cell cycle regulation, protein synthesis, and glycogen metabolism [21]. Akt could potentially regulate cell cycle progression by phosphorylating and inactivating GSK-3β, thus stabilizing nuclear β-catenin and increasing cyclin D1 transcription [22]. By inactivating GSK-3β, Akt represses GSK-3β-mediated phosphorylation and the proteolytic turnover of cyclin D1, hence increasing cyclin D1 levels in the nucleus [23]. In addition, Akt may contribute to the induction of cell cycle progression by regulating the Cdk inhibitor p27. It is known that PI3K-Akt pathway controls p27 activity and expression through two different pathways: (a) by transcriptional regulation via phosphorylating Forkhead transcription factors, it can inhibit AFX-mediated transcription of p27 [24]; and (b) posttranslationally, by directly phosphorylating p27 protein at Thr157 site [25]. To address whether cyclin D protein levels were affected by apigenin, a PI3 kinase inhibitor, cyclin D1 and cyclin D3 protein levels were determined by immunoblotting. As shown in Fig. 2A, there was a notable decrease in the steady-state levels of cyclin D1 and D3 in HER2/*neu*-overexpressing MDA-MB-453 breast cancer cells. In addition, p27 protein levels were also increased (Fig. 2A), while the

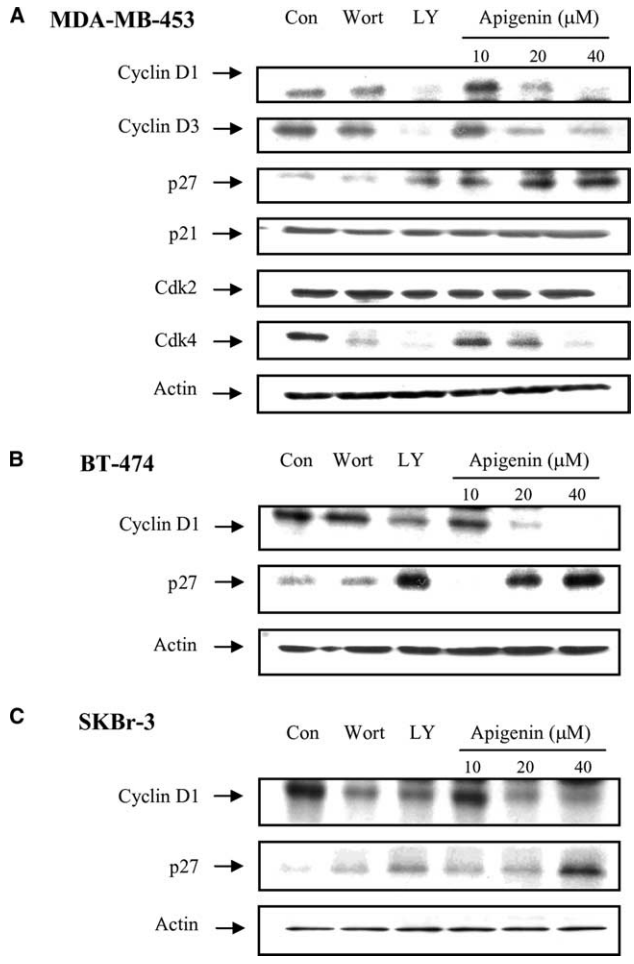


Fig. 2. Immunoblot analysis of cyclin D1, cyclin D3 and p27 proteins after apigenin treatment. (A) MDA-MB-453 cells were treated with the PI3K inhibitors wortmannin (Wort; 500 nM), or LY294002 (LY; 20 μM) or apigenin (10, 20 and 40 μM) at 37 °C for 16 h. Levels of cyclin D1, cyclin D3, p27, p21, Cdk2, Cdk4 and actin were analyzed by immunoblotting. (B) HER2/*neu*-overexpressing breast cancer cell lines BT-474 and SKBr-3 were treated with the PI3K inhibitors wortmannin (Wort; 500 nM), or LY294002 (LY; 20 μM) or apigenin (10, 20 and 40 μM) at 37 °C for 16 h. Levels of cyclin D1 and p27 were analyzed by immunoblotting.

p21 levels were not altered by any of the treatment. Wortmannin and LY294002 are known to be irreversible PI3K inhibitors, and were used here as positive controls (Fig. 2). The HER2/*neu*-overexpressing breast cancer cell lines were slightly influenced at 16 h post-treatment with wortmannin. Presumably, it is attributable to wortmannin having a relatively short half-life. To further confirm that the depletion of the cyclin D1 and p27 protein levels by apigenin is a general phenomenon, two other breast cancer cell lines that overexpress HER2/*neu* (BT-474 and SKBr-3; Fig. 2B) were analyzed and revealed similar results.

Cyclin D1 serves as the regulatory subunit of Cdk4 and contributes to its stability. Next, we assessed the effects of apigenin on Cdks protein expression. Treatment of MDA-MB-453 HER2/*neu*-overexpressing breast cancer cells with apigenin resulted in a dose-dependent decrease in protein expression of Cdk4. Nevertheless, there was no change in the protein expression of Cdk2 (Fig. 2A).

3.4. Effect of apigenin on anchorage-independent growth of breast cancer cells

Hermanto et al. [26] indicates that those human breast cancer cells with overexpression and activation of HER2/*neu* display an increased requirement for a signaling pathway mediated by PI3K-Akt in anchorage-independent growth. To determine whether apigenin may affect anchorage-independent growth, an important hallmark of the transformation phenotype, we seeded cells into soft agarose in the presence of control vehicle or varying concentrations of apigenin and monitored them for colony formation. After the treatment of apigenin, the colony-forming activity of HER2/*neu*-overexpressing breast cancer cells was significantly more suppressed than that of non-overexpressing cell lines (Fig. 3). This finding implies that HER2/*neu* is likely to be the primary target for the reduction of colony formation.

3.5. Effect of various flavonoids on HER-2/neu-overexpressing human breast cancer MDA-MB-453 cells

Flavonoids are naturally occurring plant polyphenols found in abundance in diets rich in fruit, vegetables and plant-derived beverages such as tea. Depending on their structure, some flavonoids inhibit tyrosine kinase and serine/threonine kinase activities [27]. In order to investigate the possible relationship between the chemical structure and the biological activities, we determine the relationship between chemical structures of apigenin and various flavonoids and their inhibitory activities on the protein level of HER-2/*neu* in HER-2/*neu*-overexpressing breast cancer cell lines. Nineteen flavonoids were selected for this purpose as shown in Table 1. Flavonoids are classified into several classes including flavonols, flavanones, flavanols and flavans, according to substitute at different structural position. We treated separately the HER2/*neu*-overexpressing human breast cancer MDA-MB-453 cells with 40 μM of 19 compounds at 37 °C for 24 h in the presence of serum. After treatment, the HER-2/*neu* protein was analyzed and revealed that the HER2/*neu* protein could be degraded in the presence of apigenin, luteolin, naringenin, eriodictyol and hesperetin, respectively (Fig. 4). This indicated that the position, number and substitution of the hydroxyl group of the 2-phenyl group, and the position of B ring were important for the depletion of HER2/*neu* protein by flavonoids.

4. Discussion

We have demonstrated that apigenin induces cytochrome *c* release and causes a rapid induction of caspase-3 activity and stimulates proteolytic cleavage of DFF-45 in HER2/*neu*-overexpressing breast cancer cells. In addition, apigenin inhibits colony formation, downregulates cyclin D1, D3 and Cdk4, and increases p27 protein levels in HER2/*neu*-overexpressing breast cancer cells. These results suggest that apigenin potently suppresses the growth of HER2/*neu*-overexpressing breast cancer cells, thus its therapeutic potential in advanced breast cancer is worthy of further investigation.

Many anticancer chemotherapeutic drugs exert their antitumor effect by inducing apoptosis in cancer cells. Resistance to apoptosis therefore causes a decrease in the sensitivity of can-

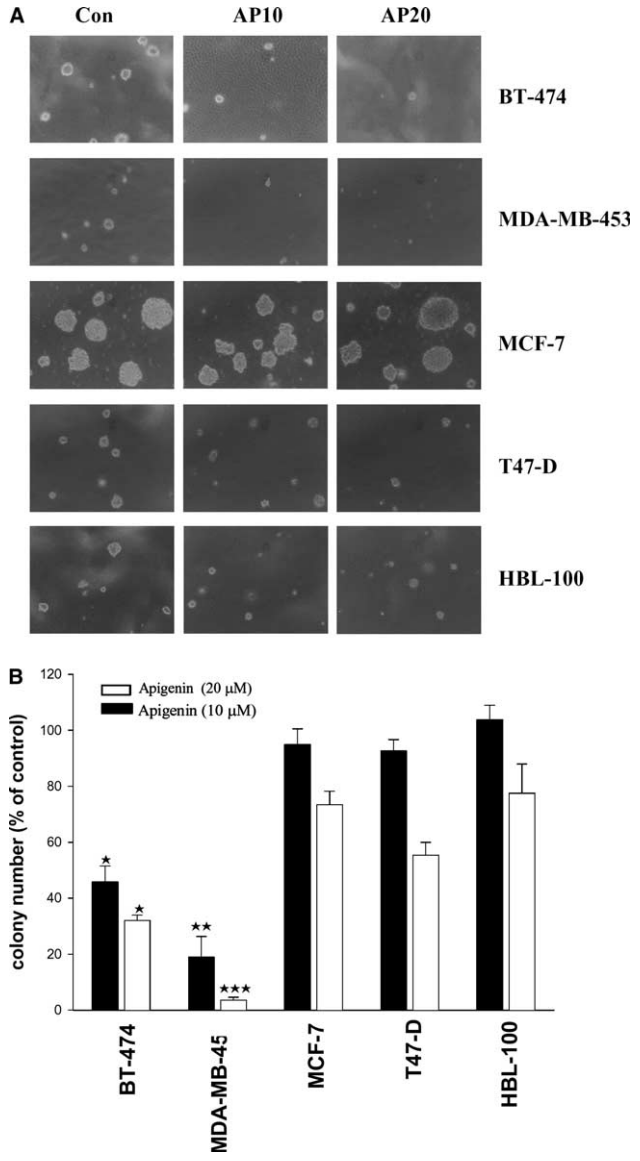


Fig. 3. Effect of apigenin on anchorage-independent growth of breast cancer cells. Cells (1×10^4 cells/well) were seeded onto 6cm dish in culture medium containing 0.35% low-melting agarose over a 0.7% agarose layer in the presence of apigenin (10, 20 μM) or vehicle control (DMSO) and were incubated for 3 weeks at 37 °C. Colonies were then stained with *p*-iodonitrotetrazolium violet (1 mg/ml) and colonies larger than 100 μm were counted. (A) A representative size distribution of BT-474, MDA-MB-453, MCF-7, T47-D and HBL-100 colonies in the presence of DMSO (Con) (left column) or 10 (middle column) and 20 (right column) μM apigenin. (B) Number of colonies in the presence of apigenin was expressed as a percentage of the number of colonies in vehicle control (DMSO). Results from three separate experiments were averaged and presented as means ± S.E. Student's *t* test was used to determine the significance of inhibition ($^{*}P < 0.01$; $^{**}P < 0.005$; $^{***}P < 0.001$).

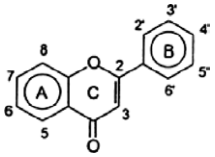
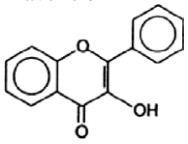
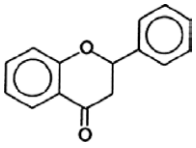
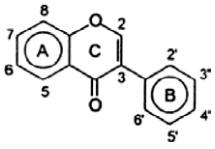
cer cells to drugs, resulting in the failure of chemotherapy. Activation of proapoptotic genes may be expected to enhance drug sensitivity in association with apoptosis that is required for the critical event of cytochrome *c* release from the mitochondria. We show here that treatment of MDA-MB-453 cells with apigenin induces the release of cytochrome *c* from mitochondria into the cytosol (Fig. 1A) followed by activating caspase-3 activities, which are believed to play a central role in mediating various apoptotic responses (Fig. 1B). These results corroborate the findings from published studies [28,29] and suggest that apigenin may enhance the susceptibility to apoptosis in HER2/*neu*-overexpressing breast cancer cells that are normally apoptosis-resistant.

By heterodimerization with HER3, HER2/*neu* can activate PI3K [30], which phosphorylates membrane phosphoinositides at the ''3'' position of the inositol ring. The NH_2-terminal pleckstrin homology domain of Akt binds PI3K-induced PIP3 in the plasma membrane, in which Akt is activated by 3-phosphoinositide-dependent kinase 1 (PDK1)-mediated phosphorylation. [31]. Once activated, Akt phosphorylates an increasing number of substrates involved in apoptosis, cell cycle regulation, protein synthesis, and glycogen metabolism. Akt can also phosphorylate and negatively regulate GSK-3β, thereby inhibiting GSK-3β-mediated phosphorylation of cyclin D1 at Thr-286. This phosphorylation accelerates proteasome-mediated degradation of cyclin D1, thus shortening its half-life [32]. Therefore, by activating PI3K/Akt and indirectly disabling GSK-3β, an excess of HER2/*neu* signals can modulate cyclin D1 and p27 and dysregulate the G1-to-S transition. Similarly, as shown recently that the anti-HER2/*neu* monoclonal antibody trastuzumab (Herceptin) can reduce cyclin D1 protein levels [33]. In addition, flavopiridol inhibits CDK activity, lowers cyclin D1 [7], and reduces erbB2 receptor proteins [8]. In our previous study, we proposed that the apigenin-induced cellular effects resulted from the loss of HER2/*neu* and HER3 expression with subsequent inactivation of PI3K and Akt in cells depending on this pathway for cell proliferation and inhibition of apoptosis [16]. Here, we demonstrate that blocking PI3k-Akt pathway can downregulate cyclin D1 and increase the expression of p27 protein. These results suggest that HER2/*neu* may regulate cellular cyclin D1 via PI3k-Akt pathway, implying that the PI3K-Akt signaling predominantly contributes to G_1-to-S progression.

The marked changes in cyclin D1/Cdk4 levels observed upon inhibition of HER2/*neu* support a critical role for this Cdk4 in HER2/*neu*-mediated cell cycle progression. Treatment of HER2/*neu*-overexpressing breast cancer cells with apigenin and the PI3K inhibitors downregulated Cdk4 protein, without altered Cdk2 protein (Fig. 2). Interestingly, our results different from recent study have shown a significant decrease in protein expression of Cdk2 and Cdk4 in hormone-refractory prostate carcinoma cells [29]. These different results with HER2/*neu*-overexpressing breast cancer cells and hormone-refractory prostate carcinoma cells suggest that unassembled Cdk4 maybe turned over more rapidly and not accumulate in HER2/*neu*-overexpressing breast cancer cells. Whether the reduction of cyclin D1 levels and/or PI3K activity can contribute to Cdk4 unassembly and destabilization requires further investigation outside the scope of this report.

The structure of the flavonoids is derived from a heterocyclic hydrocarbon, chromane, by substitution of its ring C at positions 2- or 3-with a phenyl-group (ring B) resulting in flavans, and an oxo-group at the position 4 resulting in flavanones and isoflavanones (see Table 1). Frequently, a double bond (between C2 and C3 in the ring C) is present providing these compounds with quinone-like properties. Depending on the substitution in the C-ring, these flavonoids are assigned as flavones (2-phenyl group) or isoflavones (3-phenyl group). Recent

Table 1
Chemical structures of the various flavonoids tested for the protein level of HER2/*neu* in MDA-MB-453 cells

Chemical formula	Name	Substitution							
		5	6	7	8	2′	3′	4′	5′
Flavones									
	Flavone	H	H	H	H	H	H	H	H
	5-Methoxyflavone	OCH_3	H	H	H	H	H	H	H
	7,8-Dihydroxyflavone	H	H	OH	OH	H	H	H	H
	Chrysin	OH	H	OH	H	H	H	H	H
	Apigenin	OH	H	OH	H	H	H	OH	H
	Luteolin	OH	H	OH	H	H	OH	OH	H
Flavonols									
	Galangin	OH	H	OH	H	H	H	H	H
	Kaempferol	OH	H	OH	H	H	H	OH	H
	Fisetin	H	H	OH	H	H	OH	OH	H
	Quercetin	OH	H	OH	H	H	OH	OH	H
	Myricetin	OH	H	OH	H	H	OH	OH	OH
	Rutin	OH	H	OH	H	H	OH	OH	H
Flavonol glucoside		(3: OR; R: Rhamnosylglucoside)							
Flavanones									
	Pinocembrin	OH	H	OH	H	H	H	H	H
	Naringenin	OH	H	OH	H	H	H	OH	H
	Isosakuranetin	OH	H	OH	H	H	H	OCH_3	H
	Eriodictyol	OH	H	OH	H	H	OH	OH	H
	Hesperetin	OH	H	OH	H	H	OH	OCH_3	H
Isoflavones									
	Genistein	OH	H	OH	H	H	H	OH	H
	Biochanin A	OH	H	OH	H	H	H	OCH_3	H

studies have demonstrated that, depending on their structure, flavonoids may be potent inhibitors of several kinases involved in signal transduction, mainly protein kinase C [34] and tyrosine kinases [35–37]. These studies have shown that the position and number of the hydroxyl group on the 2-phenyl ring strongly influence the conformation of the molecule and modulate their inhibitory effect. As shown previously [37], the introduction of hydroxyl groups on the flavone ring increased the inhibitory potency of the PI3-kinase of flavonoids, the polyhydroxylated flavonol, myricetin, being by far the most effective. The replacement of hydroxyl groups with methoxyl groups yielded much weaker inhibitors (diosmetin), suggesting the importance of hydrogen bonds between flavonoids and the kinase. Genistein, daidzein and genistin induce differentiation of mouse erythroleukemia cells (MEL). While biochanin A and apigenin have no such effect. These results imply that the flavonoid structure and the 4′-hydroxyl group on the 3-phenyl ring are essential for the induction of differentiation [38]. We demonstrated previously that the potency of these flavonoids towards apoptosis was: apigenin > quercetin > myricetin > kaempferol in HL-60 cells treated with 60 μM flavonoids. Thus, these findings offer a possibility that flavonoid-induced caspase-3 activation may be dependent on the number of hydroxyl groups in the 2-phenyl group and the existence of the 3-hydroxyl group [28].

In this study, we investigated the inhibitory action of 19 flavonoids of four chemical classes on the degradation of HER2/*neu* protein, a receptor which plays an important role in the development of one-third of human breast cancers. We treated the HER2/neu-overexpressing human breast cancer MDA-MB-453 cells with these 19 compounds at 37°C for 24 h. The results reveal that HER2/*neu* protein can be degraded by apigenin, luteolin, naringenin, eriodictyol and hesperetin. The IC_{50} of apigenin, luteolin, naringenin, eriodictyol and hesperetin inhibited HER2/*neu* protein expression were 12.12 ± 0.86, 4.57 ± 0.61, 47.89 ± 1.22, 45.95 ± 2.57 and 21.47 ± 1.57 μM, respectively. Interestingly, these flavonoids belong to flavones and flavanones only, while flavonals and isoflavones do not possess the ability to deplete HER/*neu* protein. The result indicates that the phenyl group at position 3 (3-phenyl group; isoflavones) and the hydroxyl group at position 3 do not have the potency to deplete HER2/*neu* protein under the concentration used in this study. These results suggest that the 2-phenyl group is essential for the degradation of HER2/*neu*. The introduction of hydroxyl group at position 4′ of 2-phenyl group increases the inhibitory potency of flavonoids. For example, the replacement of hydroxyl group with methoxyl substituent yields no inhibitory function (isosakuranetin), suggesting the importance of 4′-hydroxyl group on the 2-phenyl group. Taken together, the results of this study demonstrate that the structural features of flavonoids necessary for depleting HER2/*neu* protein are (1) the position of B ring; and (2) the existence of the 3′, 4′-hydroxyl group on the 2-phenyl group. These provide new information for the design of cancer chemopreventive agents and the study of these functional groups in the future.

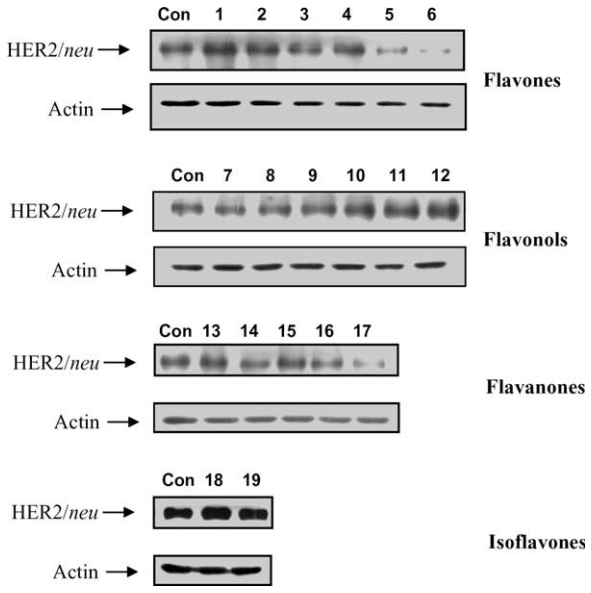


Fig. 4. Effect of various flavonoids on the expression of HER2/*neu* protein. MDA-MB-453 cells were incubated with DMSO (Con) or 1, flavone; 2, 5-methoxyflavone; 3, 7,8-dihydroxyflavone; 4, chrysin; 5, apigenin; 6, luteolin; 7, galangin; 8, kaempferol; 9, fisetin; 10, quercetin; 11, myricetin; 12, rutin; 13, pinocembrin; 14, naringenin; 15, isosakuranetin; 16, eriodictyol; 17, hesperetin; 18, genistein; 19, biochanin A (40 μM) at 37 °C for 24 h. Immunoblotting was used to measure protein levels of HER2/*neu* and actin.

Acknowledgement: This study was supported by the National Science Council NSC 91-2320-B-002-068 and NSC91-2311-B-002-037.

References

[1] Hodek, P., Trefil, P. and Stiborova, M. (2002) Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. Chem. Biol. Interact. 139, 1–21.
[2] Kawaii, S., Tomono, Y., Katase, E., Ogawa, K. and Yano, M. (1999) Antiproliferative activity of flavonoids on several cancer cell lines. Biosci. Biotech. Biochem. 63, 896–899.
[3] Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A. and McGuire, W.L. (1987) Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. Science (Wash., DC) 235, 177–182.
[4] Wang, T.C., Cardiff, R.D., Zukerberg, L., Lees, E., Arnold, A. and Schmidt, E.V. (1994) Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature (Lond.) 369, 669–671.
[5] Hortobagyi, G.N., Hung, M.C. and Buzdar, A.U. (1999) Recent developments in breast cancer therapy. Semin. Oncol. 26, 11–20.
[6] Lane, H.A., Beuvink, I., Motoyama, A.B., Daly, J.M., Neve, R.M. and Hynes, N.E. (2000) ErbB2 potentiates breast tumor proliferation through modulation of p27(Kip1)-Cdk2 complex formation: receptor overexpression does not determine growth dependency. Mol. Cell. Biol. 20, 3210–3223.
[7] Carlson, B., Lahusen, T., Singh, S., Loaiza-Perez, A., Worland, P.J., Pestell, R., Albanese, C., Sausville, E.A. and Senderowicz, A.M. (1999) Down-regulation of cyclin D1 by transcriptional repression in MCF-7 human breast carcinoma cells induced by flavopiridol. Cancer Res. 59, 4634–4641.
[8] Li, Y., Bhuiyan, M., Alhasan, S., Senderowicz, A.M. and Sarkar, F.H. (2000) Induction of apoptosis and inhibition of c-erbB-2 in breast cancer cells by flavopiridol. Clin. Cancer Res. 6, 223–229.
[9] Yu, Q., Geng, Y. and Sicinski, P. (2001) Specific protection against breast cancers by cyclin D1 ablation. Nature (Lond.) 411, 1017–1021.
[10] Kerr, J.F., Wyllie, A.H. and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer 26, 239–257.
[11] Yang, J., Liu, X. and Bhalla, K. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. Science 275, 1129–1132.
[12] Kluck, R.M., Bossy-Wetzel, E., Green, D.R. and Newmeyer, D.D. (1997) The release of cytochrome *c* from mitochondria: a primary site for bcl-2 regulation of apoptosis. Science 275, 1132–1136.
[13] Hofmann, K., Bucher, P. and Tschopp, J. (1997) The CARD. domain: a new apoptotic signaling motif. Trends Biochem. Sci. 22, 155–156.
[14] Li, P., Nijhawan, D. and Budihardjo, I. (1997) Cytochrome *c* and dATP dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91, 479–489.
[15] Kuo, M.L. and Yang, N.C. (1995) Reversion of v-H-ras-transformed NIH 3T3 cells by apigenin through inhibiting mitogen activated protein kinase and its downstream oncogenes. Biochem. Biophys. Res. Commun. 212, 767–775.
[16] Way, T.D., Kao, M.C. and Lin, J.K. (2004) Apigenin induces apoptosis through proteasomal degradation of HER2/*neu* in HER2/*neu*-overexpressing breast cancer cells via the phosphatidylinositol-3′- kinase/Akt-dependent pathway. J. Biol. Chem. 279, 4479–4489.
[17] Kim, R., Tanabe, K., Uchida, Y., Emi, M., Inoue, H. and Toge, T. (2002) Current status of the molecular mechanisms of anticancer drug-induced apoptosis. The contribution of molecular-level analysis to cancer chemotherapy. Cancer Chemother. Pharmacol. 50, 343–352.
[18] Liu, X., Zou, H., Slaughter, C. and Wang, X. (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89, 175–184.
[19] Enari, E., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391, 43–50.
[20] Sakahira, H., Masato, H. and Nagata, S. (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391, 96–99.
[21] Testa, J.R. and Bellacosa, A. (2001) AKT plays a central role in tumorigenesis. Proc. Natl. Acad. Sci. USA 98, 10983–10985.
[22] Muise-Helmericks, R.C., Grimes, H.L., Bellacosa, A., Malstrom, S.E., Tsichlis, P.N. and Rosen, N. (1998) Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway. J. Biol. Chem. 273, 29864–29872.
[23] Diehl, J.A., Cheng, M., Roussel, M.F. and Sherr, C.J. (1998) Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev. 12, 3499–3511.
[24] Brennan, P., Babbage, J.W., Burgering, B.M., Groner, B., Reif, K. and Cantrell, D.A. (1997) Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. Immunity 7, 679–689.
[25] Viglietto, G., Motti, M.L., Bruni, P., Melillo, R.M., D'Alessio, A., Califano, D., Vinci, F., Chiappetta, G., Tsichlis, P., Bellacosa, A., Fusco, A. and Santoro, M. (2002) Cytoplasmic relocalization and inhibition of the cyclindependent kinase inhibitor p27Kip1 by PKB/Akt-mediated phosphorylation in breast cancer. Nat. Med. 8, 1136–1144.
[26] Hermanto, U., Zong, C.S. and Wang, L.H. (2001) ErbB2-overexpressing human mammary carcinoma cells display an increased requirement for the phosphatidylinositol 3-kinase signaling pathway in anchorage-independent growth. Oncogene 20, 7551–7562.
[27] Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itho, N., Masabumi, S. and Fukami, Y. (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinase. J. Biol. Chem. 262, 5592–5595.
[28] Wang, I.K., Lin-Shiau, S.Y. and Lin, J.K. (1999) Induction of apoptosis by apigenin and related flavonoids through cytochrome *c* release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. Eur. J. Cancer 35, 1517–1525.
[29] Shukla, S. and Gupta, S. (2004) Molecular mechanisms for apigenin-induced cell-cycle arrest and apoptosis of hormone

refractory human prostate carcinoma DU145 cells. Mol. Carcinog. 39, 114–126.
[30] Yarden, Y. and Sliwkowski, M.X. (2001) Untangling the ErbB signalling network. Nat. Rev. 2, 127–137.
[31] Datta, S.R., Brunet, A. and Greenberg, M.E. (1999) Cellular survival: a play in three Akts. Genes Dev. 13, 2905–2927.
[32] Diehl, J.A., Cheng, M., Roussel, M.F. and Sherr, C.J. (1998) Glycogen synthase kinase-3β regulates cyclin D1 proteolysis and subcellular localization. Genes Dev. 12, 3499–3511.
[33] Yakes, F.M., Chinratanalab, W., Ritter, C.A., King, W., Seelig, S. and Arteaga, C.L. (2002) Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. Cancer Res. 62, 4132–4141.
[34] Ferriola, P.C., Cody, V. and Middleton, E. (1989) Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure–activity relationships. Biochem. Pharmacol. 38, 1617–1624.
[35] Geahlen, R.L., Koonchanok, N.M., McLaughlin, J.L. and Pratt, D.E. (1989) Inhibition of protein-tyrosine kinase activity by flavanoids and related compounds. J. Natl. Products 52, 982–986.
[36] Cushman, M., Nagarathnam, D., Burg, D.L. and Geahlen, R.L. (1991) Synthesis and protein-tyrosine kinase inhibitory activities of flavonoid analogues. J. Med. Chem. 34, 798–806.
[37] Agullo, G., Gamet-Payrastre, L., Manenti, S., Viala, C., Remesy, C., Chap, H. and Payrastre, B. (1997) Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. Biochem. Pharmacol. 53, 1649–1657.
[38] Jing, Y. and Waxman, S. (1995) Structural requirements for differentiation induction and growth-inhibition of mouse erythroleukemia cells by isoflavones. Anticancer Res. 15, 1147–1152.